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6. AUTHOR(S)

Nicholas V. Reo, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Wright State University
3640 Colonel Glenn Highway
Dayton, OH 45435

Wright State Univ.
School of Medicine
Cox Institute
3525 Southern Blvd.
Dayton, OH 45429

8. PERFORMING ORGANIZATION REPORT NUMBER

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Dr Kozumbo

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13. ABSTRACT *(Maximum 200 words)*

This AASERT grant has provided support for two graduate students in the Biomedical Sciences Ph.D. Program at Wright State University, Dayton, OH. A Ph.D. degree was conferred in 1994 to C. Goecke-Flora, while the other student, M. Adinehzadeh, currently continues in the program. These students were provided a stipend, a nominal budget for research supplies, and travel funds which enabled them to present their data at national scientific conferences. The students utilized nuclear magnetic resonance techniques in toxicology research concerning peroxisome proliferators (PPs). This work has culminated in five publications and eight published abstracts. PPs are a diverse series of chemicals with various commercial and Air Force applicabilities. Perfluorocarboxylic acids and various other halocarbons, for instance, are useful as nonflammable solvents, lubricants, and degreasers. Our research has shown that the perfluorocarboxylic acids are not metabolized, yet they produce significant effects on liver carbohydrate and phospholipid metabolism. Perfluorodecanoic acid, in particular, enhances the biosynthesis of liver phosphatidylcholine (PC), causes a significant increase in phosphocholine and diacylglycerol, and inhibits the major *de novo* pathway for the synthesis of PC. These data suggest that other pathways of phospholipid metabolism are activated following treatment with these PPs. Such effects may alter cellular signaling processes and help to explain the hepatotoxicity and carcinogenicity associated with these compounds.

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Principal Investigator: Nicholas V. Reo, Ph.D.
Institution: Wright State University, Dayton, OH

Grant Period: June 1, 1992 to May 31, 1996

Date Submitted: July 17, 1996

Executive Summary

A. Students supported by this AASERT grant:

1.	Carol Goecke-Flora	01-Jun-92 to 31-Mar-94	Ph.D. conferred June 1994; Biomedical Sciences

Ph.D. Dissertation

Carol M. Goecke-Flora, "Hepatotoxicity of Perfluorocarboxylic Acids: A ^{19}F-, ^{13}C-, and ^{31}P-NMR Investigation", Ph.D. Dissertation, Biomedical Sciences, Wright State University, Dayton, OH, 1994.

2.	Mehdi Adinehazadeh	01-Sep-94 to 31-May-96	candidate in Biomedical Sciences Ph.D. program

B. List of full publications, published abstracts, and presentations resulting from research related to this grant.

Publications:

1. N. V. Reo, L. Narayanan, K. B. Kling and M. Adinehzadeh. "Perfluorodecanoic Acid, a Peroxisome Proliferator, Activates Phospholipase C, Inhibits CTP:phosphocholine Cytidylyltransferase, and Elevates Diacylglycerol in Rat Liver". *Toxicology Letters* **86**, 1-11 (1996).

2. C. M. Goecke-Flora, J. F. Wyman, B. M. Jarnot, and N. V. Reo. "Effects of the Peroxisome Proliferator Perfluoro-*n*-decanoic Acid on Glucose Transport in the Perfused Rat Liver." *Chem. Research Toxicol.* **8** (1), 77-81 (1995).

3. N. V. Reo, C. M. Goecke, L. Narayanan, and B. M. Jarnot. "Effects of Perfluoro-*n*-octanoic Acid, Perfluoro-*n*-decanoic Acid, and Clofibrate on Hepatic Phosphorus Metabolism in Rats and Guinea Pigs *in Vivo*." *Toxicol. Appl. Pharmacol.* **124**, 165-173 (1994).

4. C. M. Goecke, B. M. Jarnot, and N. V. Reo. "Effects of the Peroxisome Proliferator, Perfluoro-n-decanoic Acid, on Hepatic Gluconeogenesis and Glycogenesis: A ^{13}C NMR Investigation." *Chem. Research Toxicol.* **7**, 15-22 (1994).

5. C. M. Goecke, B. M. Jarnot, and N. V. Reo. "A Comparative Toxicological Investigation of Perfluorocarboxylic Acids in Rats by Fluorine-19 NMR Spectroscopy." *Chem. Research Toxicol.*, **5 (4)**, 512 - 519 (1992).

Published Abstracts/Meeting Presentations:

1. M. Adinehzadeh and N. V. Reo: "Dose-Response Study of the Peroxisome Proliferator, Perfluorodecanoic Acid, on Hepatic Phospholipid Metabolism." International Society for the Study of Xenobiotics, *ISSX Proceedings*, **8**, 326 (1995). Presented at the Fourth International ISSX Meeting, Seattle, WA, August 1995.

Published Abstracts/Meeting Presentations — continued:

2. N. V. Reo, M. Adinehzadeh, and L. Narayanan: "Concentration of Liver Diacylglycerol (a Cellular Second Messenger) is Increased by the Peroxisome Proliferator, Perfluorodecanoic Acid." International Society for the Study of Xenobiotics, *ISSX Proceedings*, **6**, 281 (1994). Presented at the Sixth North American ISSX Meeting, Raleigh, NC, October 1994.

3. C. M. Goecke, N. V. Reo, J. Wyman and B. M. Jarnot: "Effects of Perfluoro-*n*-Decanoic Acid on Hepatic Glucose Transport." *The Toxicologist* **14** (1), (1994). Society of Toxicology, Annual Meeting, Dallas, TX, March 1994.

4. N. V. Reo, L. Narayanan and C. M. Goecke: "Induction of Liver Phospholipase C Activity by the Peroxisome Proliferator, Perfluorodecanoic Acid." International Society for the Study of Xenobiotics, *ISSX Proceeding*, **4**, 103 (1993). Presented at the Fifth North American ISSX Meeting, Tucson, AZ, October 1993.

5. C. M. Goecke, L. Narayanan, B. M. Jarnot and N. V. Reo: "Effects of the Peroxisome Proliferator Perfluorodecanoic Acid on Hepatic Glucose and Alanine Metabolism." International Society for the Study of Xenobiotics, *ISSX Proceeding*, **4**, 166 (1993). Presented at the Fifth North American ISSX Meeting, Tucson, AZ, October 1993.

6. N. V. Reo, C.M. Goecke, M. M. Artz, and B. M. Jarnot: "Liver Phosphorous Metabolic Response to Perfluorocarboxylic Acids and Clofibrate in Rats and Guinea Pigs: A ^{31}P NMR Study." International Society for the Study of Xenobiotics, *ISSX Proceeding*, **2**, 54 (1992). Presented at the Fourth North American ISSX Meeting, Bal Harbour, FL, November 1992.

7. C. M. Goecke, N. V. Reo, and B. M. Jarnot: "Effects of the Peroxisome Proliferator, Perfluoro-n-decanoic Acid, on Hepatic Gluconeogenesis and Glycogenesis: A ^{13}C NMR Study." International Society for the Study of Xenobiotics, *ISSX Proceeding*, **2**, 56 (1992). Presented at the Fourth North American ISSX Meeting, Bal Harbour, FL, November 1992.

8. B. M. Jarnot, C. A. Taylor, M. M. Artz, C. M. Goecke, and N. V. Reo: "Hepatic Peroxisome Induction by Perfluoro-n-decanoic Acid and Clofibrate in the Rat: Proliferation Versus Activity." International Society for the Study of Xenobiotics, *ISSX Proceeding*, **2**, 143 (1992). Presented at the Fourth North American ISSX Meeting, Bal Harbour, FL, November 1992.

C. Awards

Carol Goecke-Flora received the Graduate Student Best Poster Award at the Fifth North American Meeting of the International Society for the Study of Xenobiotics (ISSX) in Tucson, Arizona on October 17-21, 1993. This award was presented for the best scientific abstract, poster and presentation of research by a graduate student. A total of 39 student posters were evaluated by a distinguished panel of judges organized by the ISSX Officers and Council.

D. Scientific Conferences Attended by Students to Present Research Results

Mehdi Adinehazadeh

- August 1995: attended Fourth International ISSX Meeting, Seattle, WA.

Poster Presentation: M. Adinehzadeh and N. V. Reo, "Dose-Response Study of the Peroxisome Proliferator, Perfluorodecanoic Acid, on Hepatic Phospholipid Metabolism".

Scientific Conferences Attended by Students to Present Research Results — continued:

- May 1995: attended Sigma Xi Student Seminar Program, Wright State University, Dayton OH.

Oral Presentation: M. Adinehzadeh, "Dose-Response Study of the Peroxisome Proliferator, Perfluorodecanoic Acid, on Hepatic Phospholipid Metabolism".

Carol M. Goecke-Flora

- October 1993: attended Fifth North American ISSX Meeting, Tucson, AZ.

Poster Presentations:

1. N. V. Reo, L. Narayanan and C. M. Goecke, "Induction of Liver Phospholipase C Aotivity by the Peroxisome Proliferator, Perfluorodecanoic Acid."

2. C. M. Goecke, L. Narayanan, B. M. Jarnot and N. V. Reo: "Effects of the Peroxisome Proliferator Perfluorodecanoic Acid on Hepatic Glucose and Alanine Metabolism."

- November 1992: attended Fourth North American ISSX Meeting, Bal Harbour, FL.

Poster Presentations:

1. N. V. Reo, C.M. Goecke, M. M. Artz, and B. M. Jarnot: "Liver Phosphorous Metabolic Response to Perfluorocarboxylic Acids and Clofibrate in Rats and Guinea Pigs: A ^{31}P NMR Study."

2. C. M. Goecke, N. V. Reo, and B. M. Jarnot: "Effects of the Peroxisome Proliferator, Perfluoro-n-decanoic Acid, on Hepatic Gluconeogenesis and Glycogenesis: A ^{13}C NMR Study."

3. B. M. Jarnot, C. A. Taylor, M. M. Artz, C. M. Goecke, and N. V. Reo: "Hepatic Peroxisome Induction by Perfluoro-n-decanoic Acid and Clofibrate in the Rat: Proliferation Versus Activity."

E. Technical Summary of Significant Conclusions from Research

Hepatic Effects from Exposure to Perfluorocarboxylic Acids

Introduction

Perfluorocarboxylic acids and related neutral compounds have favorable chemical properties making them potentially useful in Air Force applications as nonflammable lubricants, degreasers, hydrolyic fluids, and antiwetting agents. These chemicals, however, belong to a general class of compounds known as peroxisome proliferator (PPs). Many PPs are hepatotoxic and carcinogenic; the mechanism of action is unknown and is an active area of research.

Purpose

This research project has focused on a comparative toxicological investigation of perfluoro-*n*-octanoic acid (PFOA) and perfluoro-*n*-decanoic acid (PFDA). We have employed NMR spectroscopy as a tool to study: (1) the potential biotransformation of these compounds *in vivo*, and (2) the effects that these compounds have on endogenous liver metabolism. The information provided in these studies will aid in our understanding of the biochemical mechanisms involved in the hepatotoxicity and hepatocarcinogenesis associated with PPs.

Major Results and Conclusions

1. Fluorine-19 NMR showed that **PFOA and PFDA are not metabolized in the rodent *in vivo*.** Data suggest that the toxicolgical effects are due to the parent compounds. **Differences in toxicity are believed to be related to the route and rate of clearance from the body.**

   - PFOA and PFDA show marked differences in the temporal expression of their toxicity: PFOA causes an acute lethality and transient toxicity while PFDA shows a more delayed lethality and protracted toxicity.

   - F-19 NMR spectra of liver, urine, and bile show signals from the parent compounds only; **no fluoro-metabolites are observed.**

   - F-19 NMR data show that **PFOA is readily excreted in urine, while PFDA favors biliary enterhepatic recirculation.**

2. Phosphorus-31 NMR studies show that **PFDA treatment impacts liver phospholipid metabolism.** The observed effects are unique to PFDA treatment and are not observed with PFOA or clofibrate (a classic peroxisome proliferator).

   - P-31 NMR studies of rat liver *in vivo* show signals from ATP, inorganic phosphate (P_i), phosphomonoesters (PME), and phosphodiesters (PDE).

   - **ATP concentrations are not significantly affected by treatments** with PFDA, PFOA, or clofibrate in rats and guinea pigs. These data indicate that the **doses used do not alter liver energetic viability nor do they cause significant tissue necrosis.**

   - PFDA-treated rats show an enhanced signal intensity in the PME region of the ^{31}P NMR spectrum of liver *in vivo*. This signal was identified as phosphocholine (PCho). **Liver PCho levels are significantly increased by PFDA treatment** showing a steady rise in concentration from 1 to 5 days post-dose. At 5 days posttreatment, liver [PCho] is 4-fold greater than corresponding controls (6.29 ± 0.29 *vs.* 1.76 ± 0.25 μmol/g tissue; $p < 0.05$).

   - Experiments were also conducted in guinea pigs since this species is non-responsive with regard to the proliferation of liver peroxisomes. Data showed that **the increase in liver PCho is a specific effect of PFDA treatment in rats.** It is not observed in guinea pigs, nor is it observed following treatment with various other PPs (these particular experiments only involved PFDA, PFOA, and clofibrate, but other subsequent studies have included several types of PPs). Thus this **effect on phospholipid metabolism is not correlated with peroxisome proliferation.**

   - The PFDA-induced increase in liver PCho concentration shows a dose dependent response from 5 to 50 mg/kg (30-day $LD_{50} = 42$ mg/kg). Liver PCho is significantly increased at doses ≥ 15 mg/kg ($p \leq 0.05$). The 15 mg/kg dose also causes a much lesser toxicity as evidenced by effects on the animals' food consumption and body weight. These data indicate that **the effect of PFDA on liver phospholipid metabolism is not the result of a massive toxicity but, more likely, is due to a specific metabolic effect of the compound.**

   - Studies were conducted to investigate the effects of PFOA and PFDA on specific enzymes and metabolites associated with liver phospholipid metabolism. **PFDA treatment causes a significant increase in phospholipase C (PLC) activity and a decrease in the activity of CTP:phosphocholine cytidylyltransferase (CT).** Cytosolic and microsomal PLC activities are increased 1.4- and 1.7-fold, respectively, relative to corresponding controls ($p < 0.01$). CT activities are reduced to 58% and 36% of control values in cytosol and microsomes, respectively ($p < 0.01$). **PFDA treatment also caused a 3-fold increase in liver diacylglycerol (DAG) concentration ($p < 0.01$). PFOA treatment had no effect on**

liver enzyme activities nor DAG concentration.

These data indicate that **PFDA activates the degradation of phosphatidylcholine by a phosphatidylcholine-specific PLC. Treatment also causes the inhibition of CT – the rate-limiting enzyme for the *de novo* biosynthesis of phosphatidylcholine. Thus PFDA treatment significantly impacts phosphatidylcholine turnover**. The by-product metabolites of the PLC reaction are PCho and DAG, both of which are found to be significantly elevated by PFDA treatment. These effects on phospholipid metabolism seem to be unique to PFDA treatment and are not observed following exposure to PFOA.

Phospholipid metabolism is known to play a role in cellular signaling processes. DAG is a second messenger and activator of protein kinase C. The influence of PFDA on liver phospholipid metabolism may provide a mechanism by which this compound induces various cellular responses. Indeed, it is interesting to note that PFDA is a potent peroxisome proliferator and hepatotoxicant. But unlike many other PPs (including PFOA), PFDA does not cause tumorigenesis. Its unique activity on phospholipid metabolism may hold the key to understanding the relationship between peroxisome proliferation and carcinogenesis.

3. We have initated an investigation of the **effects of various PPs on liver phospholipid content**. This work has not yet been published and, therefore, **details of the experimental procedures and results are presented in the appendix**.

   - We have refined ^{31}P NMR techniques to provide quantiative information about individual phospholipids from liver preparations.
   - **PFOA and PFDA cause a significant increase in total lipid content in the liver.**
   - Five different PPs were tested and all caused a significant increase, ranging from 29% - 55%, in total liver phospholipid content. These changes were primarily due to phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol.
   - **PFDA treatment caused a 41% and 69% increase in the liver content of PC and PE, respectively**.
   - **Our data suggest that PFDA alters the flux through liver phospholipid metabolism.** The normal *de novo* pathway for PC biosynthesis is inhibited, yet the content of PC is significantly increased following treatment. This suggests that other pathways for phospholipid biosynthesis must be activated. **Such altered metabolism may play a role in the hepatotoxic and hepatocarcinogenic effects exhibited by PPs.**

4. Carbon-13 NMR has been used to study the effects of treatment on hepatic carbohydrate metabolism.

   - **PFDA treatment caused a significant decrease in the rate of ^{13}C-glucose utilization in liver**. Apparent rates of glucose utilization were 25 - 35% lower in PFDA-treated rats (at 5 days post-dose) vs. controls.
   - **Liver glycogen synthesis from ^{13}C-labeled glucose was severely inhibited by PFDA treatment**. At 3 days post-dose glycogen deposition was significantly decreased while at 5 days post-dose glycogen synthesis was completely abolished.
   - C-13 labeled alanine was used to monitor gluconeogenesis using NMR techniques. Data showed that **the rate of alanine utilization was unaffected by PFDA treatment, and the ^{13}C label from alanine was incorporated into liver glycogen**.

     The results oulined above for the ^{13}C-glucose and ^{13}C-alanine studies show that glycogen

synthesis is inhibited when glucose serves as substrate but not when alanine is the substrate. Thus the metabolic pathway from glucose-6-phosphate (G6P) to glycogen is unaffected by PFDA treatment, and the metabolic inhibition lies at a site prior to conversion of glucose to G6P.

- Glucose transport studies were conducted in perfused liver using a radioisotope technique. Percent hepatic glucose extraction was 1.7-fold greater in control rats compared to PFDA-treated rats, 27.1 ± 3.6 vs. 15.5 ± 2.2, respectively ($p = 0.02$).

  Thus **PFDA treatment inhibits glucose uptake in liver which accounts for the inhibition observed in glycogen synthesis from glucose**. This inhibition in glucose transport activity suggests that PFDA may have a major impact on membrane structure/function.

  The carbohydrate and phospholipid studies both indicate that **PFDA has some interaction at the plasma membrane. This effect alters phospholipid metabolism, disrupts membrane strucure/function, and may potentiate other effects through cellular signaling processes.**

- With ^{13}C-alanine as substrate, the NMR experiment also provides information about the tricarboxylic acid (TCA) cycle activity. In particular, the **entry of pyruvate into the TCA cycle via pyruvate carboxylase (PC) or pyruvate dehydrogenase (PDH) can be distinquished and the relative activity of these two metabolic pathways can be determined**. Data showed that **PFDA-treated rats yeild a significantly greater flux through the PDH pathway in comparison to controls. This result may reflect a dysfunction in fatty acid metabolism due to treatment**. The greater PDH activity in PFDA-treated rats suggests that pyruvate serves as the predominant source of acetyl-CoA. In pair-fed controls (rats fasted for about 3 days), the acetyl-CoA is provided through fatty acid oxidation and thus PDH activity is low. In PFDA-treated rats, however, PDH activity may be greater because fatty acid oxidation and ketogenesis are inhibited.

APPENDIX — unpublished data

Analyses of liver phospholipid profiles by ^{31}P NMR. Since these results have not yet been published, data are displayed below.

Methods

We have refined and further developed NMR methods for the absolute quantitation of individual liver phospholipids. We are preparing a manuscript describing these methods which will be submitted to *Magnetic Resonance in Medicine*. Briefly, tissue lipids were prepared using a modified Folch extraction method. Livers were homogenized in chloroform:methanol (2:1 v/v) and the chloroform phase was separated and dried. A sample was prepared for NMR analyses by reconstituting the dried lipids in a mixed solvent of chloroform-d, methanol, and aqueous Cs_2EDTA.

Results

Figure 1 shows a ^{31}P NMR spectrum of a rat liver lipid extract and depicts the signals from individual phospholipids. The chemical shift of the peaks (resonance frequency) is temperature sensitive and, thus, the spectral resolution is affected by temperature. We have measured the temperature dependence of the individual resonances to provide the information necessary to optimize spectral resolution. These data are shown in Figure 2.

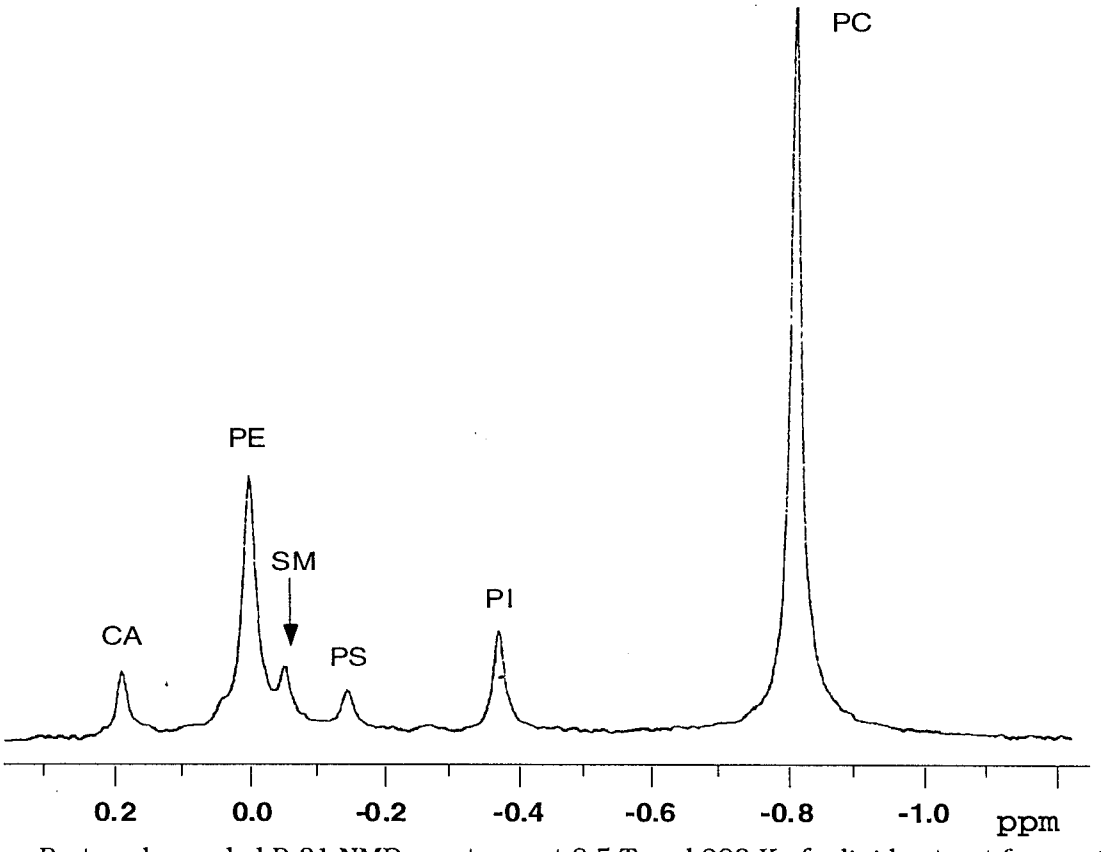

Figure 1 : Proton-decoupled P-31 NMR spectrum at 8.5 T and 293 K of a lipid extract from rat liver. Individual phospholipids are: PC, phosphatidylcholine; PE, phosphatidylethanol-amine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; and CA, cardiolipin.

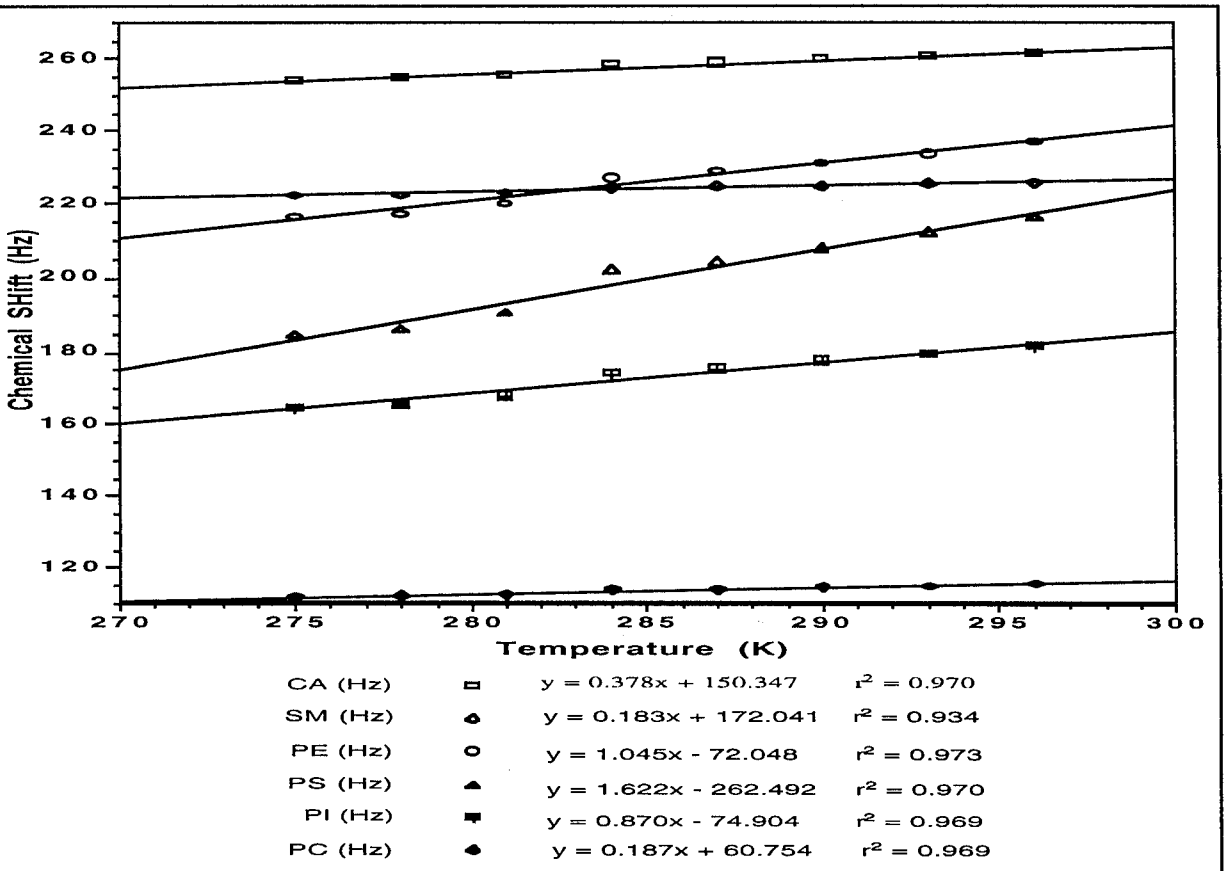

Figure 2 : Plot showing the temperature dependence of the chemical shifts for individual phospholipid resonances from the P-31 spectrum. Data were fit to linear equations which are shown below the plot along with the correlation functions.

The spectral peak areas are proportional to concentration. The spectrum can provide absolute quantitation of individual phospholipids when calibrated against a standard. We have used an external standard (phosphocreatine solution) and analyzed liver samples for phospholipid content. Table 1 displays the results from rats that were fasted for 12 hours prior to liver extraction. These data represent ~96% of all phospholipids and compare favorably with literature values in which phospholipids were determined by chromatography techniques.

TABLE 1
Percentage of rat liver phospholipids (mean ± SD; n = 3)
determined from P-31 NMR data.

Phospholipids	Liver Content
PC	56.2 ± 1.9 %
PE	20.2 ± 0.5 %
PS	4.4 ± 1.0 %
PI	8.1 ± 0.9 %
CA	2.7 ± 0.2 %
SM	4.5 ± 0.5 %
Total	25.0 ± 0.6 μmol/g liver

Five different PPs (PFOA, PFDA, clofibrate, DEHP, and Wy-14,643) were investigated with regard to their effects on liver phospholipids. Male F-344 rats were separated into two groups and administered either a single PP compound (treatment group) or vehicle solution (control group). Treatment protocols matched those previously reported to produce maximal hepatic effects. Food consumption and body weights were recorded daily; controls were pair-fed to corresponding treated rats.

Total liver lipid content for treated and control groups is shown in Figure 3. PFOA and PFDA treatment caused a 31% and 42% increase in total lipid content relative to corresponding controls, respectively ($p \leq 0.04$). Other PPs did not significantly affect lipid content.

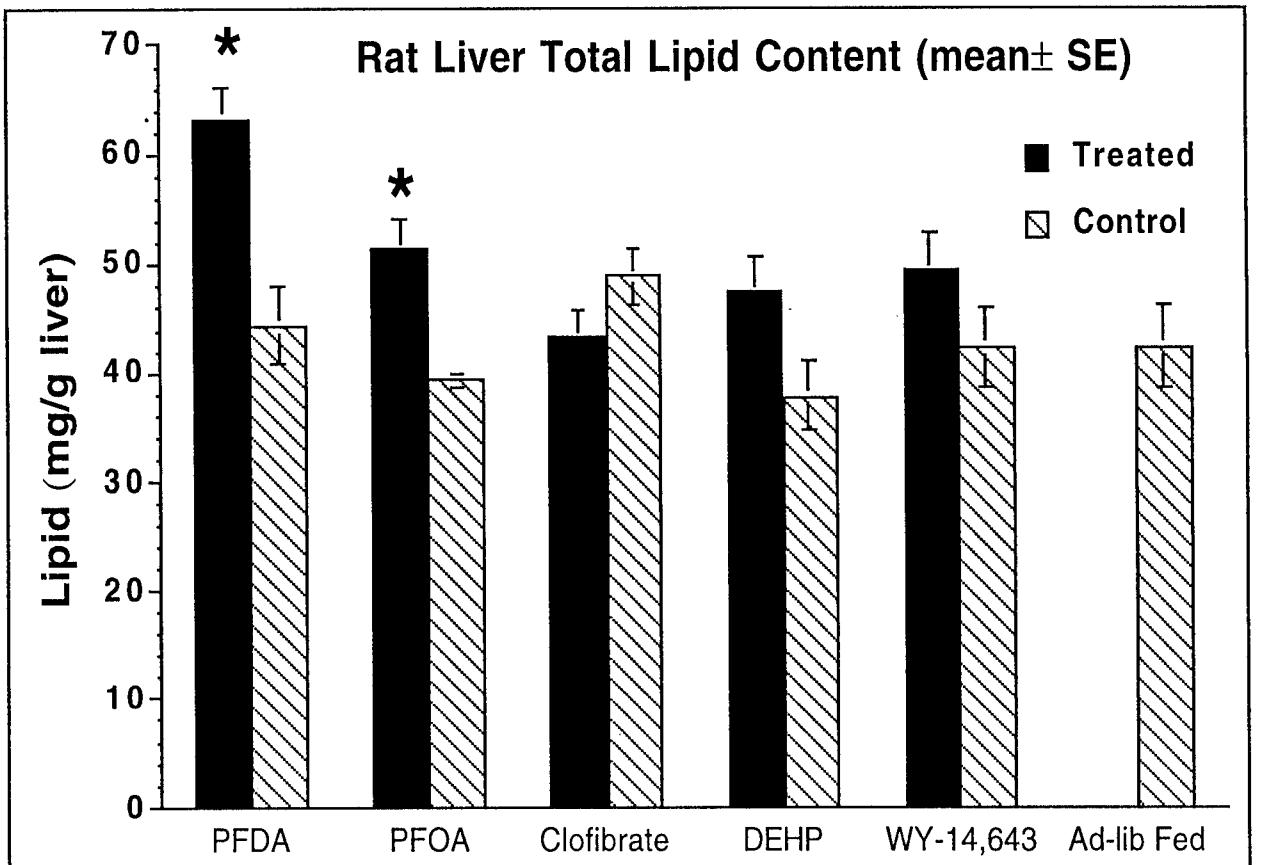

Figure 3 : Total lipid content (mean ± SE; n = 4 - 6) measured in livers from rats treated with various peroxisome proliferators and corresponding pair-fed controls. An additional control group in which animals were given *ad-libitum* access to food is also included for comparison.

Spectral data were analyzed for individual phospholipids and Figure 4 shows the mean phospholipid content (µmol/g liver) for all groups (n = 4 - 6). All treatment groups show a significant increase in total phospholipid content ranging from 29% - 55% which is mainly due to PC, PE, and PI. Wy-14,643 and DEHP treatments also cause a ~1.5-fold increase in CA. Ad-libitum fed controls show significantly less phospholipids than all other groups which demonstrates the influence of nutritional status on hepatic phospholipid content. Interestingly, our previous experiments have shown that PFDA induces degradation of hepatic PC and inhibits its *de novo* biosynthesis. Since these current data show that PC content has increased following PFDA treatment, it suggests that other pathways for PC synthesis must be active.

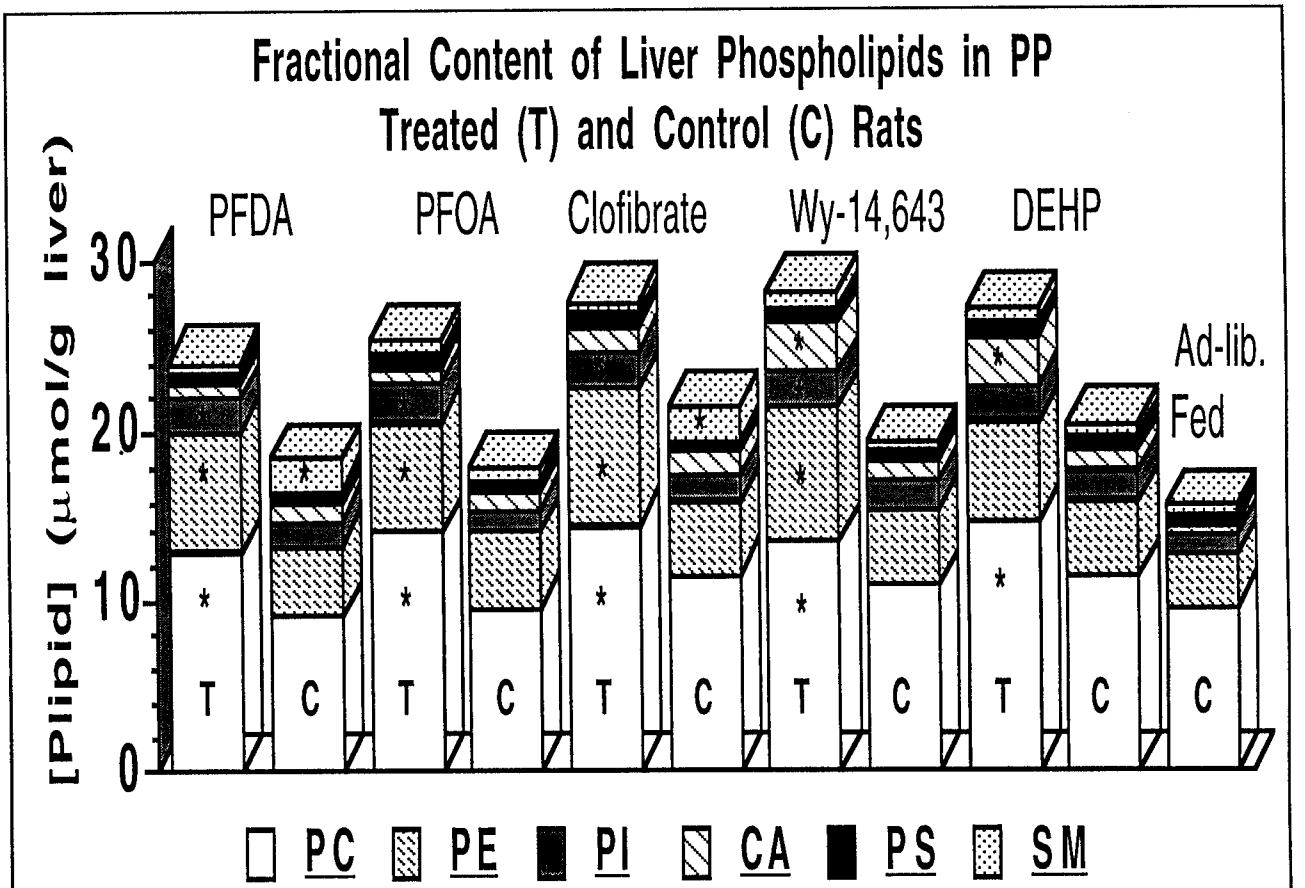

Figure 4 : Bargraph showing the mean content of liver phospholipids from PP-treated (T) and corresponding control (C) groups. Each bar depicts the fraction of individual phospholipids. Asterisks denote a statistical difference in comparison with corresponding pair-fed controls ($p \leq 0.05$).

These data provide new insights about the influence of PPs on liver phospholipid metabolism. Peroxisome proliferator-induced changes in phospholipid content and composition may correlate with their degree of toxicity and/or carcinogenic potential. Further studies which focus on the specific metabolic pathways of phospholipid metabolism may help to elucidate the mechanisms by which PPs initiate various cellular events that can profoundly impact tissue viability.